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Rapid report

Stoichiometry of cholesterol–sphingomyelin condensed complexes in monolayers

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Abstract

Some binary mixtures of cholesterol and phospholipids in monolayers have thermodynamic phase diagrams with two upper miscibility critical points. This feature has been interpreted in terms of 'condensed complexes' between the phospholipid and cholesterol. The present work gives evidence for the formation of complexes with a common simple integral stoichiometry in binary mixtures of cholesterol and a series of five sphingomyelins where the amide-linked acyl chain length is varied. This indicates that these complexes have a distinct geometry even though they form a liquid phase.

Keywords: Critical point; Phase diagram; Membrane; Liquid mixture; Immiscibility

Phospholipid–cholesterol mixtures have been extensively investigated as models of the lipid bilayer regions of animal cell membranes [1,2]. Studies of these mixtures in lipid monolayers have been used due to their simplicity and the ease with which the molecular density can be varied through changes of applied pressure. At lower pressures many mixtures of phospholipids and cholesterol form two coexisting liquid phases [3–5]. These liquid phases typically form micrometer size liquid domains that are readily observed using epifluorescence microscopy. The coexisting phases generally merge into one phase at higher monolayer pressures. The appearance and disappearance of the coexisting liquid phases as a function of pressure and monolayer composition permit the determination of thermodynamic phase diagrams for the mixtures. Phase diagrams have provided valuable clues as to molecular interactions between cholesterol and phospholipids, particularly evidence for the formation of 'condensed complexes', as discussed below [6]. The coexisting liquid phases seen in monolayers are sometimes found at pressures where the average molecular density is somewhat below the molecular densities of the same lipid mixtures in bilayers and biological membranes. Nonetheless, the complexes deduced from the phase diagrams persist at higher pressures where the molecular densities are comparable to those in bilayers and biological membranes.

A number of binary mixtures of cholesterol with phospholipids having two unsaturated fatty acid chains have particularly simple phase diagrams, each having a single, well defined upper miscibility critical point [7]. On the other hand, a number of

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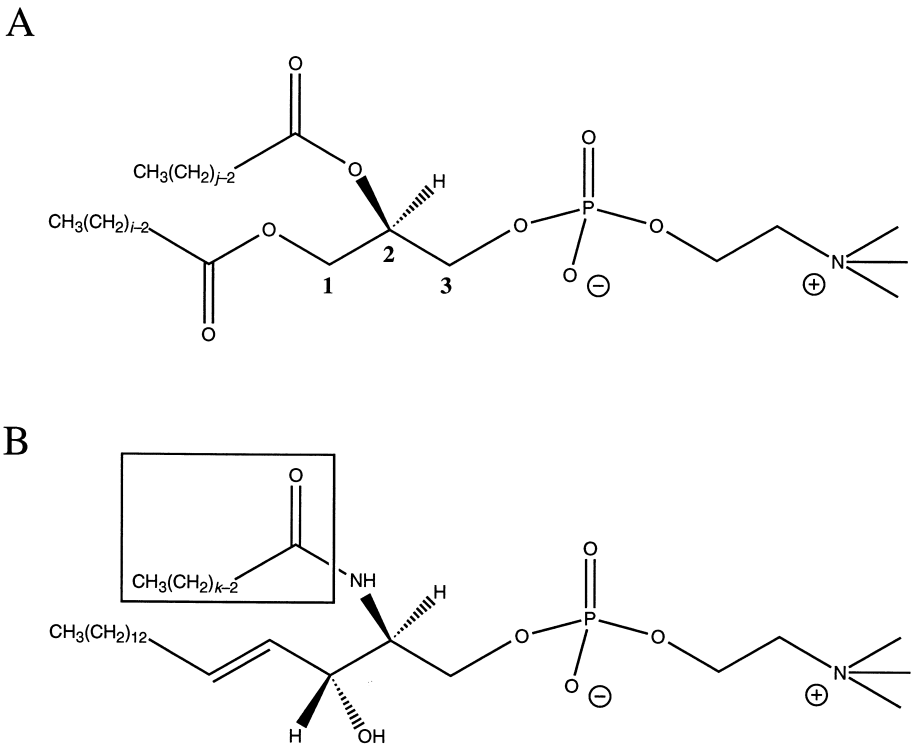

Fig. 1. Glycero- and sphingophospholipids. (A) Structure of a typical phosphatidylcholine (PC) molecule. The three carbons of the glycerol backbone are labelled. The phosphorylcholine headgroup moiety is ester-linked to carbon 3. Two fatty acid chains of lengths i and j, referred to as the $sn1$ and $sn2$ acyl chains, are ester-linked to carbons 1 and 2 respectively. The notation i:0-j:0 PC is used when referring to these molecules, where 0 refers to the number of unsaturations in the acyl chain. (B) Structure of a typical SM molecule. The sphingosine backbone serves as one of the hydrocarbon chains. The phosphorylcholine headgroup moiety is once again ester-linked, and a single acyl chain of variable length k (boxed) is amide-linked to the sphingosine backbone. The notation k:0 SM is used when referring to these molecules.

mixtures of cholesterol with phospholipids having saturated fatty acid chains show highly unusual phase diagrams having two upper miscibility critical points [6]. These unusual phase diagrams have been interpreted in terms of complex formation between cholesterol (C) and phospholipid (P),

$$nq\mathrm{C} + np\mathrm{P} \leftrightarrow \mathrm{C}_{nq}\mathrm{P}_{np} \quad (1)$$

Here p and q are stoichiometry integers, and n is an oligomerization parameter reflecting the cooperativity of this complex formation. These complexes are referred to as condensed complexes because (a) the observed average molecular area is often a sharp minimum (cusp) at the complex stoichiometry, and (b) the complex formation is cooperative. The stoichiometry of the complexes is inferred from the compositions at cusps in the phase diagrams as well as at cusps in plots of average molecular area vs. composition. The cusps in physical properties sometimes occur near 33% cholesterol, suggesting $q=1$, $p=2$. This stoichiometry has been suggested in many earlier studies of bilayers [8–10]. However, in a number of monolayer experiments cusp compositions are found elsewhere in the range 25–43% [6]. Although the cusps in physical properties are often sharp, they are never sharp enough to establish unambiguously an integral stoichiometry.

The present work was stimulated by the observation that sphingomyelins (SMs) form complexes with cholesterol [11], and by the possibility of preparing a series of five SMs in which only the composition of the amide-linked fatty acid chain is varied. Structures for the SMs and related glycerophospholipids are shown in Fig. 1.

We find that all these SMs form complexes with cholesterol, and that they all have essentially the

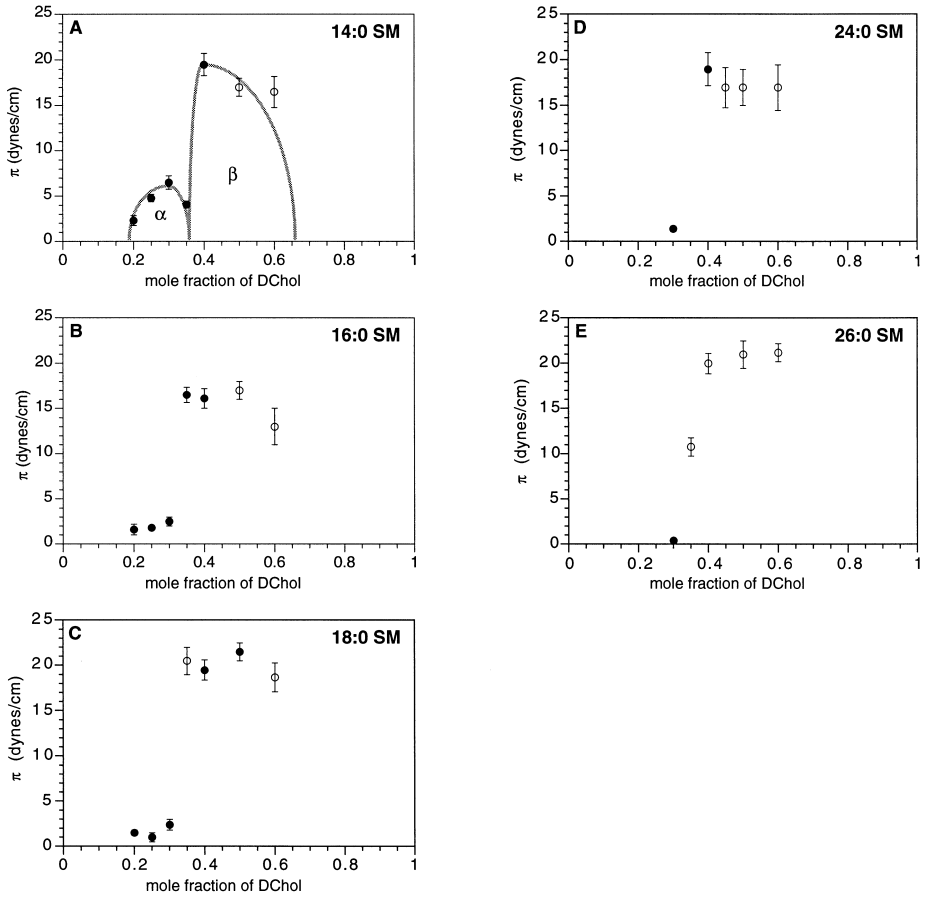

Fig. 2. Phase diagrams showing liquid–liquid miscibility critical points for mixtures of DChol and SMs of differing acyl chain length. Plotted data points represent transition pressures where two liquid phases disappear into a single homogeneous phase. Stripe superstructure phases, which represent proximity to a critical point, were observed at the transitions marked by filled circles and not at those marked by open circles. The two-phase coexistence region corresponding to low DChol mole fractions is referred to as α and the two-phase coexistence region corresponding to high DChol mole fractions is referred to as β The phase diagrams in A–E are for mixtures of DChol with 14:0, 16:0, 18:0, 24:0, and 26:0 SM respectively. The error bars represent deviations during three independent phase boundary measurements.

same cusp composition, consistent with $q=1$, $p=2$. Even though no attempt is made to determine n, it is clear from the data and previous results [11] that the formation of these complexes is cooperative with n likely in the range 3–5. Taken together, these results support the view that these cusp compositions do represent integral stoichiometries for SM–cholesterol mixtures. As in previous studies most experiments have been carried out using dihydrocholesterol (DChol) rather than cholesterol, so as to minimize air oxidation. In all experiments controls are carried out to ensure that results with cholesterol itself are essentially the same. For simplicity the text generally refers to cholesterol except when describing specific results obtained with DChol.

SMs containing homogeneous acyl chains were synthesized by reacylating sphingosylphosphorylcholine (lyso-SM) with the *N*-hydroxysuccinimide (NHS) ester of the desired fatty acid [12,13]. Briefly, NHS derivatives were synthesized and recrystallized and then reacted with purified lyso-SM, which had been produced by deacylating egg SM using methanolic HCl. The resulting SM was purified by flash column chromatography (Silica gel, 200–400 mesh, Aldrich) and recrystallized from $CHCl_3/CH_3OH$ using −20°C acetone. SM purity and homogeneity were confirmed

by thin layer chromatography and by capillary gas chromatography, respectively.

Cholesterol and DChol were obtained from Sigma (St. Louis, MO, USA). A fluorescent dye, Texas Red dihexanoylphosphatidylethanolamine (TR-DHPE, Molecular Probes, Eugene, OR, USA), which is preferentially excluded from the DChol- or cholesterol-rich phase, was used to provide contrast between the liquid phases. Lipid mixtures with 0.2 mol% of TR-DHPE were spread from a 1 mg/ml chloroform solution onto the air–water interface of a Teflon trough which had a movable barrier to change the surface pressure. Epifluorescence microscopy methods described previously [5,6] were used for the phase diagram measurements. The experiments were carried out with Ar-saturated water in the subphase and in a chamber flooded with Ar to minimize air oxidation of the SMs. Most of the experiments were carried out with DChol instead of cholesterol to minimize artifacts due to cholesterol oxidation. In previous work we have shown that the phase behavior of both sterols in mixtures with phospholipids are similar [14]. Several of the phase diagram measurements in this study were repeated with cholesterol instead of DChol and yielded similar values for the transition pressures. In cases where the domains formed were very small (less than 1 μm in diameter), an electric field was used to fuse the domains to improve their observability. The electric field was turned off during subsequent phase transition measurements. All of the phase transition pressures represent the point at which liquid domains nucleated from a uniform one-phase fluid background. Average molecular area measurements were carried out by adding aliquots of lipids until a specified surface pressure was reached.

Fig. 2A–E shows phase diagrams of binary mixtures of DChol and 14:0, 16:0, 18:0, 24:0, and 26:0 SM. All of the phase diagrams exhibit two two-phase coexistence regions with a sharp cusp in between. As discussed previously, such phase diagrams of binary mixtures can be explained by a thermodynamic model where DChol or cholesterol (C) and phospholipids such as SM (P) form a condensed complex $C_{nq}P_{np}$ [6]. The two two-phase coexistence regions are labeled α and β in order of increasing DChol concentration. The α two-phase region arises from immiscibility between the complex and SM, whereas the β two-phase region arises from the immiscibility between the complex and DChol. The α region features circular liquid domains that are 5–10 μm in diameter, while the β region features tiny white liquid domains that are approximately 1–2 μm in diameter. In some cases in the β region, an electric field was used to fuse these domains to improve their observability. At the transition pressure, the two liquid phases merge into one homogeneous liquid phase. This transition is sometimes accompanied by the formation of a stripe phase (indicative of proximity to a critical point [15]). The cusp in the phase diagram between the α and β regions correlates with the stoichiometry of the complex. Note that the concentration of DChol at which the cusp occurs remains the same as the SM acyl chain length is varied from 14 to 26 carbons.

Fig. 3A–E shows average molecular area measurements for the binary mixtures corresponding to Fig. 2A–E. In all cases, there is a minimum in average area/molecule at a composition corresponding to that of the cusp in the respective phase diagrams. This cusp is sharper for some of the SM chain lengths than the others. Binary mixtures of DChol and a SM with a singly unsaturated amide-linked acyl chain (24:1 SM) were also observed using epifluorescence microscopy, but poor contrast made it very difficult to observe domains or phase transitions.

The cusp compositions found in the present work are in the range 0.35 ± 0.05 for binary mixtures of cholesterol and five different SMs. The results are then consistent with the formation in each case of a specific complex, with $q = 1$, $p = 2$. As noted above, we have not attempted to determine the cooperativity (n) of formation of these complexes, but comparison with earlier calculations and phase diagrams indicates that n is likely to be in the range 3–5 [11]. Thus, the number of molecules in the cooperative unit is of the order 9–15. Our data then imply that the complexes have a definite molecular structure, with perhaps a distribution of sizes but not compositions.

The argument in favor of a single, defined molecular structure is indirect. If the experimental data were sufficiently accurate to prove that the cusp compositions were equal to the ratio of integral numbers, then the argument for a fixed molecular geometry would be compelling. However, the data are not that accurate for a given binary mixture. Nonethe-

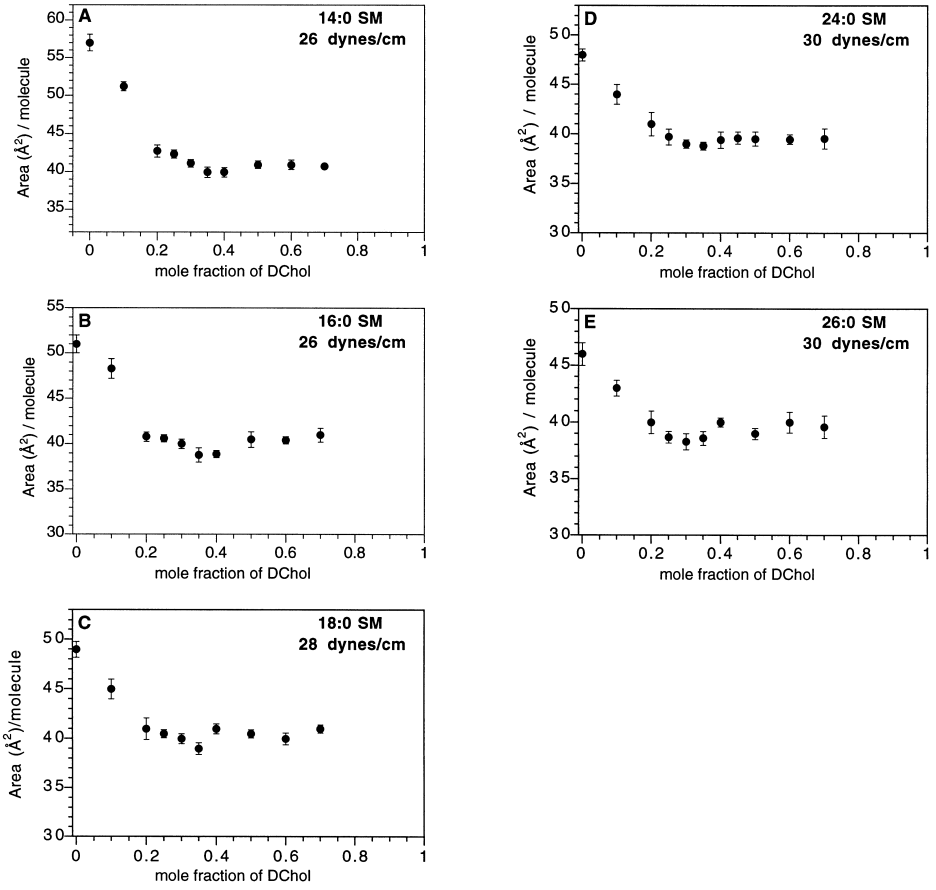

Fig. 3. A–E show average molecular area measurements for mixtures of DChol with 14:0, 16:0, 18:0, 24:0 and 26:0 SM respectively. In all the mixtures, the area measurements are conducted at a pressure well above both upper miscibility critical points. Error bars represent deviations during three independent measurements.

less, since five binary mixtures of related phospholipids have yielded closely similar cusp compositions, the integral numbers for the stoichiometry of the complexes appear very likely to us.

Cusp compositions found for binary and ternary mixtures of glycerophospholipids with cholesterol are in the range 25–43% [6,11,14]. It is clear that the cusp compositions depend in a sensitive way on the chemical structure of the phosphatidylcholine. For example, there is a large difference in cusp composition for mixtures of DChol with a 14:0-16:0 PC and with a 16:0-14:0 PC [16]. Although all the results are consistent with the formation of complexes with different integral stoichiometries, the question remains open.

Essentially all of the previously proposed lattice-like models of cholesterol–phospholipid mixtures use integral stoichiometries to account for physical properties [17–24]. (Here 'integral stoichiometry' is taken to signify a composition derived from the ratio of integral numbers.) Some of these integral stoichiometries are based on a hexagonal lattice and an assumed equivalence of the two fatty acid chains of the phospholipids. As an additional concern, it may be noted that a Penrose tiling of the plane with two unsymmetrical objects would lead to stoichiometries that are not integral [25]. Based on chemical experience we believe that the condensed complexes are likely to have integral stoichiometries but there is no a priori way to be sure of this.

The stoichiometry reported in the present work is in no way inconsistent with the $q=2$, $p=1$ compositions reported by Huang et al. for phosphatidylcholine–cholesterol mixtures. In these mixtures, at the $q=2$, $p=1$ composition, the chemical activity of cholesterol increases to the point that cholesterol monohydrate forms [24,26]. Condensed complexes with

$q = 1$, $p = 2$ stoichiometries may nonetheless be found in these mixtures at the lower cholesterol concentrations [6]. There is no reason to believe that a given binary mixture cannot form complexes with more than one stoichiometry [22,27] at different membrane compositions.

The relation between molecular structure and putative complex stoichiometries is not clear. A number of lattice-like structural models for a $q = 1$, $p = 2$ stoichiometry have been proposed [8–10]. In many of these models the fatty acid chains have been treated as equivalent. Since cholesterol and phospholipids are molecules with no symmetry, it is possible that the *sn*1 and *sn*2 chains of glycerophospholipids may have distinguishable interactions with cholesterol. In previous work it was suggested that the *sn*1 fatty acid chain is the more significant for determining stoichiometry [16]. That suggestion is compatible with the results of the present work, and an earlier suggestion that the sphingosine chain of SMs is to be correlated with the *sn*1 fatty acid chain of glycerophospholipids [28,29]. That is, in the present study significant variations in the length of the amide-linked fatty acid chain have shown no effect on the cusp compositions, and inferred stoichiometry.

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